



Melatonin elevates intracellular free calcium in human platelets by inositol 1,4,5-trisphosphate independent mechanism

Sharda Kumari, Debabrata Dash *

Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

ARTICLE INFO

Article history:

Received 16 May 2011

Accepted 27 May 2011

Available online 7 June 2011

Edited by Veli-Pekka Lehto

Keywords:

Melatonin
Intracellular calcium
Platelets
SERCA
SOCE

ABSTRACT

Several studies have indicated the existence of direct effects of melatonin on platelets. Here we show that, melatonin at high concentration is capable of significantly raising platelet intracellular calcium even in the absence of an agonist. The effect of melatonin on platelets was abolished by luzindole, a melatonin receptor blocker, and rotenone, while it was unaffected by cell-permeable antagonists of either inositol 1,4,5-trisphosphate (IP₃) receptor, phospholipase C (PLC), or bafilomycin A1, which discharges acidic calcium stores. Melatonin-induced manganese entry provided evidence for activation of bivalent cation entry. Thus, our data suggest that melatonin evoked the elevation of platelet intracellular calcium through depletion of mitochondrial Ca²⁺ stores and store-operated calcium entry (SOCE), while the action was independent of the PLC-IP₃ axis.

© 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Intracellular calcium regulates major platelet functions including shape change, spreading, adhesion, aggregation and finally thrombus formation [1–3]. Platelet agonists increase cytosolic [Ca²⁺] by two mechanisms: release of Ca²⁺ from intracellular stores and entry of extracellular Ca²⁺ through plasma membrane channels. Store-operated calcium entry (SOCE) is a process controlled by calcium concentration in platelet dense tubular system (DTS), the analogue of sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER). Depletion of DTS calcium stores triggers the activation of SOCE channels, in the plasma membrane [4]. Besides, mitochondria have also been implicated in maintaining cellular Ca²⁺ homeostasis [5–8]. Recent works of Rosado have provided compelling evidence in favor of platelet acidic organelles, including lysosomes and lysosome-related organelles, as additional Ca²⁺ stores [9,10].

Melatonin (N-acetyl 5-methoxytryptamine) is a hormone secreted by the pineal gland in brain, which regulates biological rhythm. Several studies have implicated existence of direct effects of melatonin in peripheral organs like platelets. Brain is not directly accessible to observation in human beings when alive. Moreover, studies on cerebrospinal fluid are subject to ethical consideration. Neurons and platelets share common structure and functional characteristics that have led researchers to use platelets as 'peripheral' model of neurons [11]. It is reported that

melatonin at physiological level (less than 1 μM) potentiates aggregation, whereas at concentrations higher than 10 μM, it attenuates aggregation induced by agonists like collagen, ADP and epinephrine [12–14] involving cyclooxygenase pathway [15]. Melatonin exerts biological actions through the activation of two G protein-coupled receptors, MT1 and MT2 [16,17]. In addition, melatonin interacts with intracellular proteins such as calmodulin, calreticulin or tubulin and antagonizes the binding of Ca²⁺ to calmodulin [18]. Melatonin binding sites have been characterized in platelets from guinea pig [19] as well as human origin [20]. Besides its well recognized central action on circadian biological rhythm, melatonin has also been shown to modulate activities of cardiovascular system and regulate platelet function. [12]. A recent study by Rosado and his colleagues demonstrated that, melatonin implants enhance thrombin evoked calcium flux in platelets from vaccinated sheep [21]. Effect of melatonin on intracellular cAMP accumulation reported in nerve tissues or cell lines transfected with melatonin receptors were mostly inhibitory. However, potentiating actions on adenylate cyclase have also been reported [22]. In human platelets, melatonin has inhibitory effect on prostacyclin-stimulated cAMP accumulation in vitro [13].

Melatonin supplements help prevent jet lag, particularly in people who cross five or more time zones. It may also help with sleep problems associated with menopause. Gels, lotions, or ointments containing melatonin may protect against sunburn and other skin damage. In this study, we report for the first time that pharmacological dose of melatonin elevates intracellular free calcium in human platelets. Rise in cytosolic Ca²⁺ is central to platelet activation, which triggers signaling events leading to thrombus

* Corresponding author. Fax: +91 542 2367568.

E-mail address: ddass@satyam.net.in (D. Dash).

formation and cardiovascular disorders including myocardial infarction and stroke [5,23]. Bach et al. have shown involvement of second messengers, inositol 1,4,5-trisphosphate (IP_3) and $[Ca^{2+}]_i$ in the signaling induced by melatonin in rat insulinoma INS1 cells [24]. Recently, it has been revealed that exogenous melatonin releases Ca^{2+} from intracellular stores in exocrine pancreatic cell lines [25]. We show here that, melatonin-induced rise in $[Ca^{2+}]_i$ in platelets was not precluded by cell-permeable antagonists of IP_3 or phospholipase C (PLC), signifying a signaling mechanism unrelated to IP_3 -PLC signaling axis. Capacitative cation influx was confirmed from melatonin-induced entry of extracellular $MnCl_2$ into platelets. Our results also indicated that melatonin evoked rise in $[Ca^{2+}]_i$ in platelets pretreated with bafilomycin A1, which discharges acidic stores, but not with rotenone that releases Ca^{2+} from mitochondrial stores. These findings suggested that melatonin elicited rise in platelet cytosolic calcium by stimulating release from mitochondrial stores, as well as stimulating SOC entry.

2. Materials and methods

Fura 2/AM was obtained from Calbiochem. 2-APB (2-aminoethoxydiphenyl borate), U73122, luzindole, dibutyl cAMP, melatonin, EGTA, thapsigargin, rotenone, bafilomycin A1, $MnCl_2$ and aspirin were the products of Sigma.

2.1. Platelet Preparation

Blood from healthy volunteers, who had denied taking medication in the previous 2 weeks, was collected in citrate phosphate dextrose and centrifuged at 180 g for 10 min. The supernatant (platelet-rich plasma or PRP) was incubated with 1 mM acetylsalicylic acid for 15 min at 37 °C. After addition of EDTA (5 mM), PRP was centrifuged at 600 g for 10 min. Cells were washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4 and 1 mM EGTA, supplemented with 5 mM glucose). Platelets were finally suspended in buffer B which was same as buffer A but without EGTA. The final platelet count was adjusted to $3-4 \times 10^8$ /ml. Handling and mixing was done gently in order to prevent activation of platelets.

Wherever indicated, platelets were preincubated at 37 °C for 10 min with following inhibitors: U-73122 (10 μ M), 2-APB (100 μ M), luzindole (500 μ M) and dibutyl cAMP (5 mM) etc.

2.2. Measurement of intracellular free calcium

PRP was incubated with 2 μ M Fura-2 AM for 45 min at 37 °C in the dark. The Fura 2-loaded platelets were washed and resuspended in buffer B at 1×10^8 cells/ml. Fluorescence was recorded

in 400 μ l aliquots of platelet suspensions, both control as well pretreated with melatonin, at 37 °C under non-stirring condition. Excitation wavelengths were 340 and 380 nm and emission wavelength was set at 510 nm in Hitachi fluorescence spectrophotometer (model F-2500). Changes in intracellular free calcium concentration, $[Ca^{2+}]_i$, upon treatment with thrombin and melatonin were monitored from the fluorescence ratio (340/380) using Intracellular Cation Measurement Program in FL Solutions software. F_{max} was determined by lysing the cells with 40 μ M digitonin in the presence of saturating $CaCl_2$. F_{min} was determined by the addition of 2 mM EGTA. Intracellular free calcium was calibrated according to the derivation of Grynkiewicz et al. [26].

2.3. Statistical methods

Standard statistical methods were employed. Parametric methods (*t* test) were used for evaluation and significance tests were considered significant at *P* less than 0.05 (2-tailed tests). Data were presented as means \pm SD of at least five individual experiments from different blood donors.

3. Results

3.1. Melatonin elevates free intracellular Ca^{2+} in human platelets

As platelet activation is associated with increase in the concentration of cytosolic calcium [27], we examined effect of melatonin on intracellular calcium flux. Incubation of platelets with melatonin at concentration below 100 μ M did not affect basal calcium level in resting human platelets. However, rise in melatonin concentration led to dose-dependent increase in level of intracellular calcium. To understand whether the observed rise in cytosolic Ca^{2+} has extracellular or intracellular origin, we performed the experiment either in presence of 1 mM calcium or 1 mM EGTA, followed by addition of melatonin (500 μ M) (Fig. 1). When extracellular Ca^{2+} was chelated by EGTA, melatonin induced a minor increase in $[Ca^{2+}]_i$ from 100 nM to 200 nM, indicative of release from intracellular stores. However, presence of 1 mM extracellular Ca^{2+} evoked a stronger rise in cytosolic Ca^{2+} from 140 nM to 400 nM consistent with entry from external source.

3.2. PLC- IP_3 axis is not involved in melatonin-induced calcium rise

Intracellular mobilization of calcium in DTS of platelets is mediated through IP_3 receptors [28]. So, we asked whether PLC- IP_3 axis was responsible for the rise in calcium in response to melatonin in absence of extracellular Ca^{2+} . We reasoned that, melatonin treatment might induce activation of platelet PLC leading to

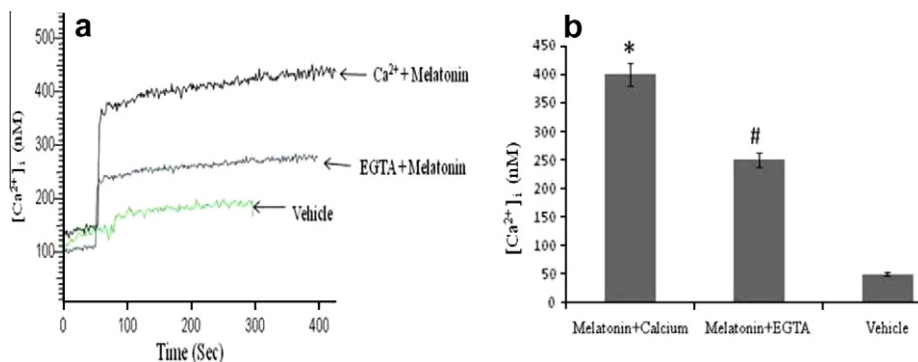


Fig. 1. Melatonin raises free intracellular Ca^{2+} in human platelets. (a) Fura 2-loaded platelets were preincubated for 5 min with either 1 mM calcium or 1 mM EGTA, followed by addition of 500 μ M melatonin. Intracellular calcium was measured as described. (b) The bar diagram shows an average of five independent experiments. (**P* < 0.05 as compared to melatonin induced platelets in EGTA set; #*P* < 0.05 as compared to vehicle)

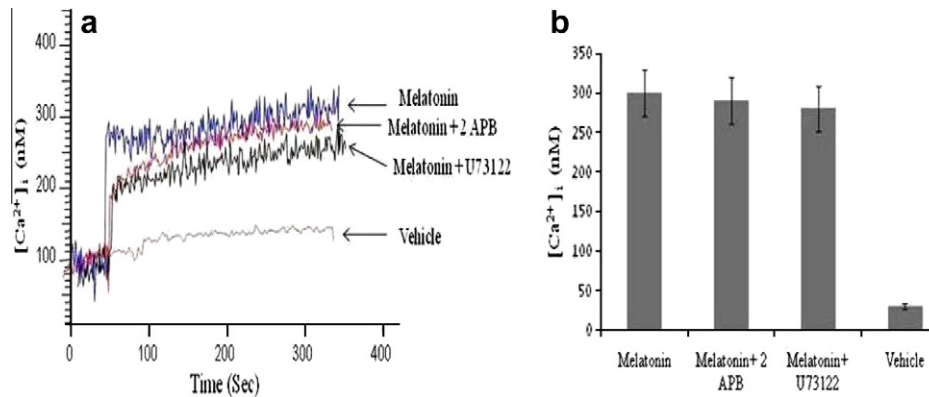


Fig. 2. PLC-IP₃ axis is not involved in melatonin-induced rise in cytosolic calcium. (a) Fura 2-loaded platelets were incubated for 10 min with either 2-APB or U73122, in presence of 1 mM EGTA, followed by addition of 500 μ M melatonin. Intracellular calcium was measured as described. (b) The bar diagram shows an average of five independent experiments.

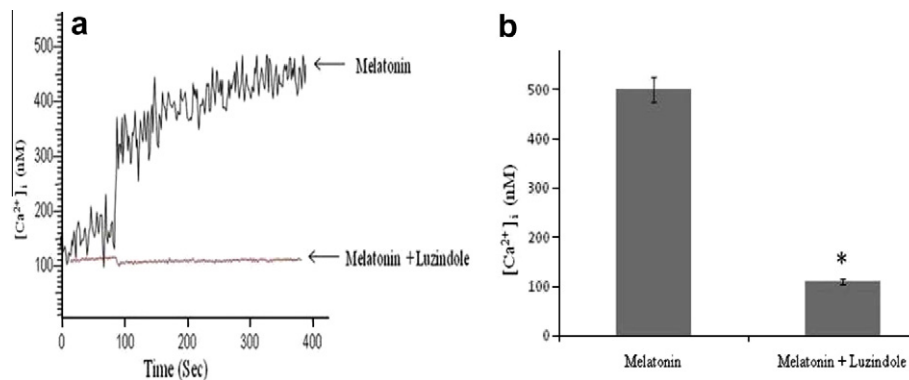


Fig. 3. Rise in intracellular Ca^{2+} by melatonin was attenuated by luzindole, a melatonin receptor antagonist. (a) Fura 2-loaded platelets were incubated for 10 min with luzindole (500 μ M) followed by stimulation with melatonin (500 μ M). (b) The bar diagram shows an average of five independent experiments (* $P < 0.05$ as compared to melatonin-induced platelets).

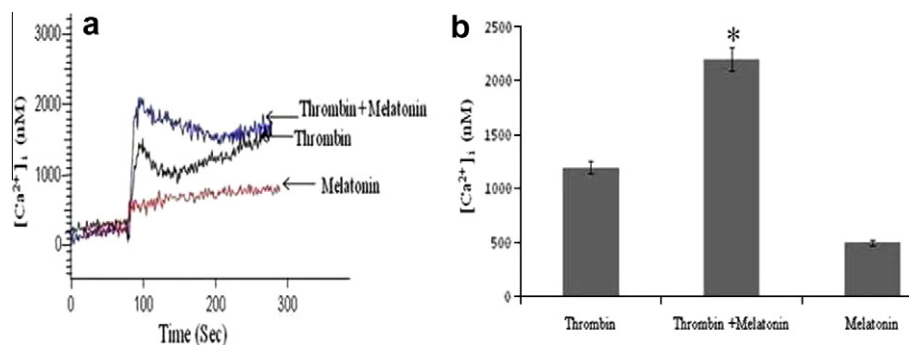


Fig. 4. Melatonin potentiated thrombin-induced rise in intracellular calcium. (a) Fura 2-loaded platelets were incubated for 10 min with melatonin (500 μ M) followed by stimulation with thrombin (1 IU/ml). (b) The bar diagram shows an average of five independent experiments. (* $P < 0.05$ as compared to thrombin-induced platelets)

generation of IP₃ and mobilization of calcium from intracellular stores.

Platelets were separately incubated with 2-APB (100 μ M) (an IP₃ antagonist), U73122 (10 μ M) (inhibitor of PLC β) or the vehicle, followed by addition of melatonin (500 μ M). EGTA was included in the suspension medium to prevent calcium entry from extracellular source. None of the inhibitors could influence the action of melatonin on platelet calcium (Fig. 2), thus ruling out the involvement of PLC-IP₃ pathway as a possible underlying mechanism.

3.3. Rise in platelet intracellular Ca^{2+} by melatonin is attenuated by luzindole, a melatonin receptor antagonist

Next we asked whether melatonin evoked rise in intracellular calcium is by interaction with specific receptors on platelet surface. Platelets were incubated with luzindole (500 μ M), a melatonin receptor antagonist, followed by addition of the hormone in presence of 1 mM extracellular calcium. Melatonin-induced rise in cytosolic calcium was found to be abolished completely by

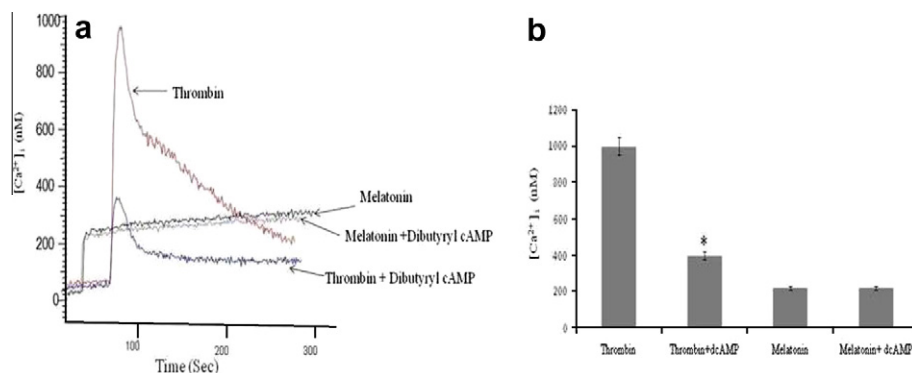


Fig. 5. Effect of melatonin on platelets was not regulated by level of cytosolic cAMP. (a) Fura 2-loaded platelets were incubated for 10 min with dibutyl cAMP (5 mM) followed by treatment with melatonin (500 μ M) or thrombin (1U/ml). (b) the bar diagram shows an average of five independent experiments (* $P < 0.05$ as compared to thrombin-induced platelets).

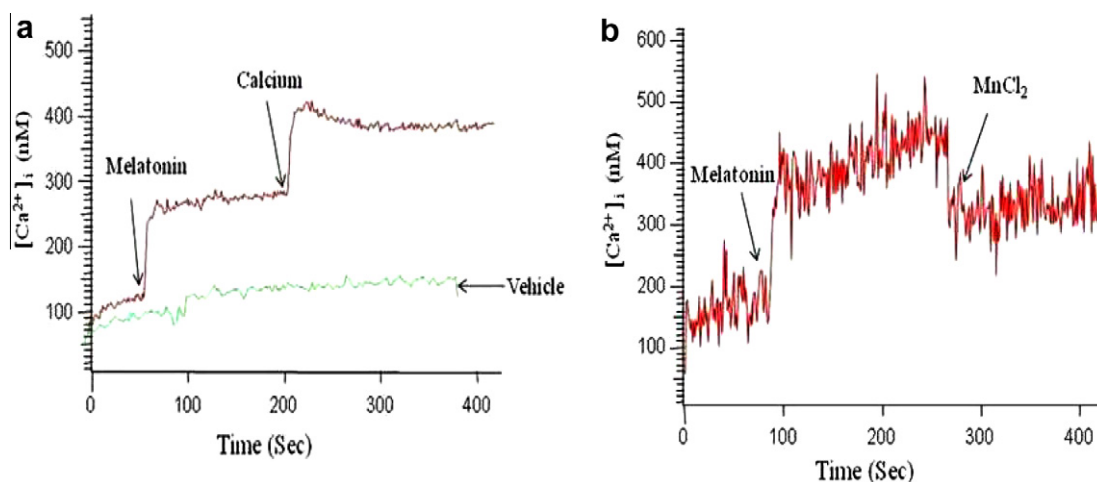


Fig. 6. Melatonin exhibited thapsigargin-like properties. (a) Melatonin (500 μ M) was added to Fura 2-loaded platelets, followed by addition of 1 mM calcium after 150 s. (b) Melatonin (500 μ M) was added to Fura 2-loaded platelets, followed by addition of 2 mM $MnCl_2$ after 200 s. Fluorescence ratio was measured as described. Traces shown are representative of at least five separate experiments.

luzindole (Fig. 3), suggesting that observed effects of melatonin were receptor-dependent.

3.4. Melatonin potentiates thrombin-induced rise in intracellular calcium

In order to enquire any synergistic effect of melatonin with thrombin, a physiological agonist of platelets, cells were first incubated with the hormone (500 μ M) followed by thrombin (1 IU/ml) in presence of extracellular calcium, and cytosolic calcium flux was studied. As expected, thrombin alone induced significant increase in intracellular $[Ca^{2+}]_i$. Melatonin was found to significantly potentiate the effect of thrombin (Fig. 4). The initial rise in calcium and the following plateau were nearly 2 times higher in melatonin-pretreated platelets than in presence of thrombin alone. This observation was consistent with the earlier report that melatonin reduced lag phase and increased calcium mobilization in thrombin-activated sheep platelets [21].

3.5. Effect of melatonin on platelets is not regulated by level of cytosolic cAMP

Melatonin is known to decrease cAMP level in target cells [29]. In platelets decrease in cytosolic cAMP is associated with

significant enhancement in agonist-induced platelet activation and rise in intracellular calcium [30]. Hence, we next asked whether increasing level of intracellular cAMP will attenuate melatonin effect on cytosolic calcium in platelets. When platelets were

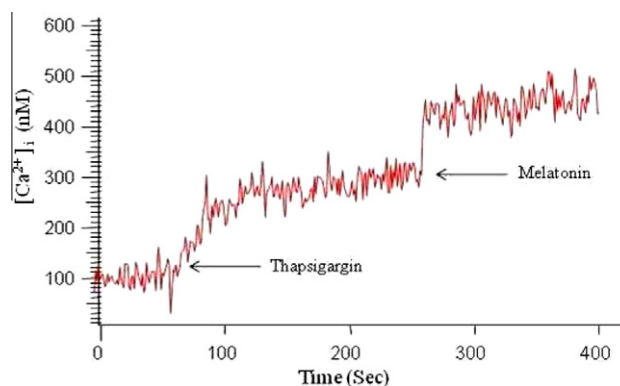


Fig. 7. Melatonin-induced calcium rise was not associated with inhibition of SERCA. Thapsigargin (1 μ M) was added to Fura 2-loaded platelets, followed by addition of melatonin (500 μ M). Fluorescence ratio was measured as described. Traces shown are representative of at least five separate experiments.

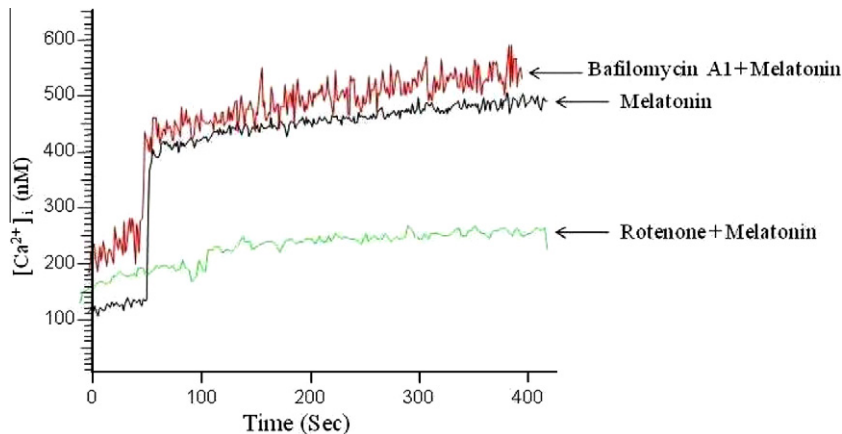


Fig. 8. Cytosolic Ca^{2+} flux in melatonin-treated platelets was regulated by mitochondrial source of calcium and was independent of acidic stores. Fura 2-loaded platelets were incubated for 10 min either with bafilomycin A (1 μM) or with rotenone (20 μM), followed by addition of melatonin (500 μM). Fluorescence ratio was measured as described. Traces shown are representative of at least five separate experiments.

pre-incubated with dibutyl cAMP (5 mM), a cell-permeable analog of cAMP, for 10 min at room temperature, thrombin-mediated platelet aggregation (not shown) or rise in intracellular calcium (Fig. 5) was completely inhibited. However, dibutyl cAMP pre-treatment did not affect melatonin-induced rise in cytosolic calcium (Fig. 5).

3.6. Stimulation of SOC entry by melatonin

As stated earlier, melatonin elicited moderate increase in platelet intracellular Ca^{2+} in IP_3 -independent manner. When cells were suspended in Ca^{2+} -free medium, subsequent addition of 1 mM CaCl_2 to the medium brought about a further strong rise in intracellular calcium indicative of SOCE (Fig. 6a). Thus, it could be surmised that melatonin depleted intracellular Ca^{2+} stores in a manner resembling the action of thapsigargin [31] and led to stimulation of SOCE.

The external cation influx was further examined in the presence of Mn^{2+} . The ion penetrates across plasma membrane of stimulated platelets to bind with fura-2, which results in quenching of fura-2 fluorescence. When MnCl_2 was added to platelet suspension 1 min after melatonin, decrease in fluorescence was recorded (Fig. 6b), consistent with bivalent cation entry activated by melatonin.

3.7. Melatonin-induced calcium rise is not associated with inhibition of SERCA

In order to understand whether the observed effects of melatonin were attributable to suppression of SERCA activity, we pre-incubated platelets with high dose of thapsigargin (1 μM) to inhibit the SERCA isoforms, namely SERCA2b (on platelet DTS) and SERCA3 (on acidic Ca^{2+} store) [10], followed by addition of melatonin. As expected, thapsigargin elicited a rise in intracellular calcium, which was further augmented in presence of melatonin (Fig. 7). This ruled out the possibility that melatonin-induced rise in cytosolic Ca^{2+} could be brought about by inhibition of SERCA isoforms by the hormone.

3.8. Melatonin induces release of Ca^{2+} from mitochondria and not from acidic organelles in platelets

As melatonin-induced calcium rise was not associated with inhibition of SERCA and IP_3 -mediated pathway ruling out involvement of DTS, we asked whether acidic organelles might be the source of calcium releasable by melatonin. We pre-incubated

platelets with bafilomycin A1 (1 μM), inhibitor of vacuolar type H^+ -ATPase that releases calcium from acidic granules, followed by addition of melatonin (Fig. 8). Our results indicated that preincubation with bafilomycin A1 did not alter Ca^{2+} release induced by melatonin.

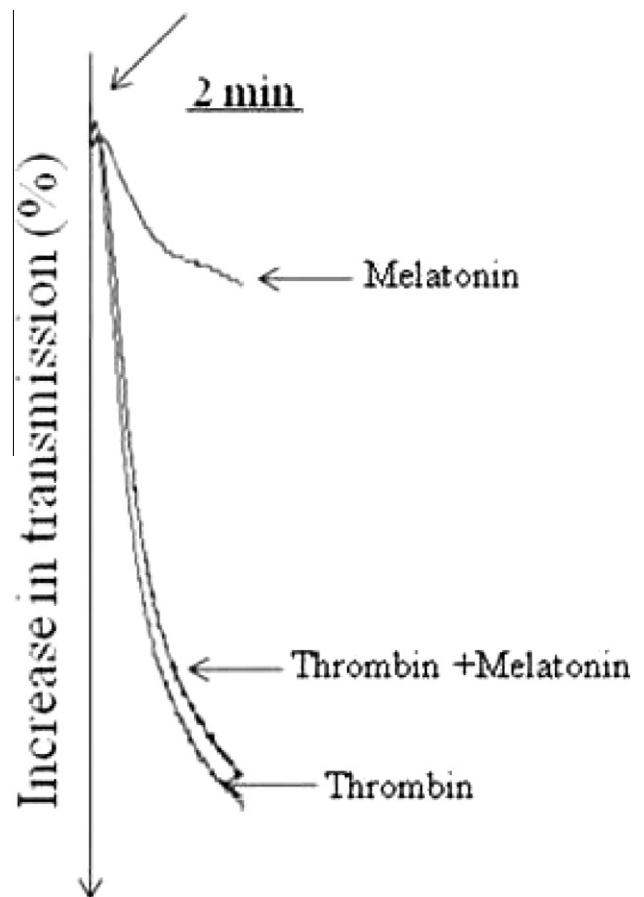


Fig. 9. Melatonin had no effect on thrombin-mediated platelet aggregation. Platelets were pre-incubated with either melatonin (500 μM) or vehicle for 15 min under stirring, followed by addition of thrombin (0.5 U/ml) in certain experiments, as indicated. Traces shown are representative of at least five separate experiments.

Next, in order to implicate mitochondrial source of Ca^{2+} in melatonin action, we performed the experiments in the presence of rotenone (20 μM) that dissipates mitochondrial transmembrane potential and releases Ca^{2+} from the organelle. Subsequent addition of melatonin did not bring about further increase in $[\text{Ca}^{2+}]_i$, thus indicative of mitochondrial contribution to calcium rise elicited by melatonin (Fig. 8). However, signaling path linking melatonin to depletion of mitochondrial calcium store remains unknown.

3.9. Melatonin has no effect on thrombin-induced platelets aggregation

In order to examine the effect of melatonin on thrombin-mediated platelet aggregation, thrombin (0.5 U) was added to platelets pretreated with melatonin or vehicle. Thrombin evoked robust aggregation in control platelets. There was no change in the aggregation profile when platelets were pre-incubated with melatonin (Fig. 9). However, melatonin alone was also found to elicit minor wave of platelet aggregation (Fig. 9).

4. Discussion

In the present study, we show that melatonin alone, at higher concentration reachable by pharmacological doses, induced rise in intracellular calcium in resting platelets, as well as potentiated the effect of thrombin in raising platelet calcium but had no effect on thrombin-induced platelet aggregation. Platelets elevate cytosolic calcium by release from compartmentalized intracellular stores or by facilitating entry from outside through plasma membrane channels. In resting cells low cytosolic calcium is maintained by action of different Ca^{2+} -ATPases and exchangers in synergy, which act by Ca^{2+} sequestration into the stores and extrusion across the plasma membrane [32]. SERCA isoforms present on the membranes of DTS as well as acidic Ca^{2+} stores are responsible for store refilling that in turn results in termination of SOCE [10,3].

Effect of melatonin on platelet cytosolic calcium was completely abolished in presence of luzindole (Fig. 3), a known antagonist of melatonin receptor, which was suggestive hormone acting through its receptors on platelet membrane. Although melatonin is known to lower cAMP in target cells, raising cytosolic cAMP by its non-hydrolyzable analog did not modify the effect of melatonin on platelets, thus ruling out participation of this nucleotide in the specific signaling pathway. Melatonin could raise cytosolic calcium either by inducing release from intracellular stores or facilitating entry from extracellular milieu. In our experiment, melatonin-induced rise in calcium was significantly reduced by chelation of extracellular calcium by EGTA. To test whether PLC was stimulated in melatonin-treated platelets leading to generation of IP_3 and subsequent release of calcium from IP_3 -sensitive stores, we separately pre-incubated platelets with antagonists of PLC and IP_3 receptor, followed by treatment with melatonin. None of the inhibitors could influence the action of melatonin on platelet calcium (Fig. 2), therefore ruling out the involvement of PLC- IP_3 axis as a possible mechanism. Melatonin was found to induce rise in $[\text{Ca}^{2+}]_i$ even after platelet SERCA isoforms were inhibited by thapsigargin (Fig. 7), thus excluding the role SERCA inhibition in melatonin function. Melatonin evoked rise in $[\text{Ca}^{2+}]_i$ in platelets pretreated with bafilomycin A1 but not with rotenone, which was consistent with major contribution of mitochondrial calcium stores in eliciting the melatonin effect. Melatonin-induced manganese entry in platelets was suggestive of activation of bivalent cation entry by the hormone. Taken together, both mitochondria and SOCE contributed to melatonin-induced calcium increase in platelets.

Melatonin is often prescribed in therapeutic form to ease insomnia, combat jet lag, protect cells from free-radical damage and boost immune system [33]. It has been demonstrated to be effective in the treatment of many circadian rhythm sleep disorders [34] and used as a medication for sleep disturbance in depression, treatment of psychiatric disorders and as a skin protector for ultraviolet light [35]. Melatonin has also been shown to be effective in fibromyalgia [36]. Short term side effects of the therapy include vivid, frightening nightmares, headaches and mild depression, along with morning grogginess and confusion [37]. In a recent clinical study a dose as high as 2 mg extended-release melatonin has been administered every night to insomniac patients for a period of 3 weeks [38]. Melatonin is considered a dietary supplement and mostly available as an alternative medicine without formal prescription and dosage regulation. As with all non-regulated supplements, there has been no substantial study to document adverse effects of melatonin from its long term use or from intake of higher dose. So our present study should add a cautionary note to unchecked and unregulated melatonin therapy.

Earlier reports have suggested inhibitory effects of melatonin on agonist-induced platelet aggregation and secretion [12]. However, we show here that, melatonin, at higher concentration reachable by therapeutic doses, is capable of significantly raising platelet intracellular calcium even in absence of any agonist, while it synergizes the effect of platelet agonists. Recently, it has been shown that exogenous melatonin elevates $[\text{Ca}^{2+}]_i$ in pancreatic AR42 J cells, which facilitates release of digestive enzymes downstream of physiological secretagogues [24]. We show here that, melatonin-induced rise in $[\text{Ca}^{2+}]_i$ in platelets was not prevented by cell-permeable antagonists of IP_3 or PLC, suggestive of signaling mechanism unrelated to IP_3 -PLC axis. Our results supported that melatonin released calcium from the mitochondrial source that is known to play minor role in intracellular calcium homeostasis in platelets. As platelet is a critical player in arterial thrombosis leading to ischemic pathologies like coronary heart disease and stroke, and intracellular calcium being a major determinant of platelet reactivity, any therapeutic use of melatonin should be monitored under intense caution.

Author contributions

S.K. performed different experiments; D.D. designed the research, supervised the whole work and analyzed results; D.D. and S.K. wrote the paper.

Conflict of interest

The authors declare no conflicting interests.

Acknowledgement

This research was supported by grants received by D. Dash from the Department of Biotechnology (DBT), the Department of Science and Technology (DST) and the council of Scientific and Industrial Research (CSIR), Government of India. S. Kumari is a recipient of research fellowship from the CSIR.

References

- [1] Jackson, W.S., Nesbitt, S. and Kulkarni, J. (2003) Signaling events underlying thrombus formation. *J. Thromb. Haemost.* 1, 1602–1612.
- [2] Croce, K., Flaumenhaft, R., Rivers, M., Furie, B., Furie, B.C., Herman, I.M. and Potter, D.A. (1999) Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J. Biol. Chem.*, 36321–36327.
- [3] Harper, A.G.S., Mason, M.J. and Sage, S.O. (2009) A key role for dense granule secretion in potentiation of the Ca^{2+} signal arising from store-operated calcium entry in human platelets. *Cell Calcium* 45, 413–420.

- [4] Parekh, A.B. and Putney, J.W. (2005) Store operated calcium channels. *J. Physiol. Rev.* 85, 757–810.
- [5] Collins, T.J., Berridge, M.J., Lipp, P. and Bootman, M.D. (2002) Mitochondria are morphologically and functionally heterogeneous within cells. *EMBO J.* 21, 1616–1627.
- [6] Gilibert, J.A., Bakowski, D. and Parekh, A. (2001) Energized mitochondria increase the dynamic range over which inositol 1,4,5-trisphosphate activates store-operated calcium influx. *EMBO J.* 20, 2672–2679.
- [7] Villalobos, C., Nuñez, L., Montero, M., Garcia, A.G., Alonso, M.T., Chamero, P., Alvarez, J. and Garcia-Sancho, J. (2002) Redistribution of Ca^{2+} among cytosol and organelle during stimulation of bovine chromaffin cells. *FASEB J.* 16, 343–353.
- [8] González, A., Granados, M.P., Salido, G.M. and Pariente, J.A. (2003) Changes in mitochondrial activity evoked by cholecystokinin in isolated mouse pancreatic acinar cells. *Cell Signal.* 15, 1039–1048.
- [9] Rosado, J.A. (2010). Acidic Ca^{2+} stores in platelets. *Cell Calcium.* 1–7.
- [10] Zbidi, H., Jardin, I., Woodard, G.E., Lopez, J.J., Berna-Erro, A., Salido, G.M. and Rosado, J.A. (2011) STIM1 and STIM2 are located in the acidic Ca^{2+} stores and associates with Orai1 upon depletion of the acidic stores in human platelets. *J. Biol. Chem.* 14, 12257–12270.
- [11] Morera, A.L. and Abreu, P. (2005) Existence of melatonin in human platelets. *J. Pineal Res.* 39, 432–433.
- [12] Kornblihtt, L.L., Finocchiaro, L. and Molinas, F.C. (1993) Inhibitory effect of melatonin on platelet activation induced by collagen and arachidonic acid. *J. Pineal Res.* 14, 184–191.
- [13] Del Zar, M.M., Martinuzzo, M., Falcón, C., Cardinali, D.P., Carreras, L.O. and Vacas, M.I. (1991) Inhibition of human platelet aggregation and thromboxane B^2 production by melatonin. Correlation with plasma melatonin levels. *J. Pineal Res.* 11, 135–139.
- [14] Cardinali, D.P., Del zar, M.M. and Vacas, M.I. (1993) The effects of melatonin in human platelets. *Acta Physiol. Pharmacol. Ther. Latinoam.* 43, 1–13.
- [15] Martinuzzo, M., Del Zar, M.M., Cardinali, D.P., Carreras, L.O. and Vacas, M.I. (1991) Melatonin effect on arachidonic acid metabolism to cyclooxygenase derivatives in human platelets. *J. Pineal Res.* 11, 111–115.
- [16] Sethi, S., Adams, W., Pullock, J. and Witt-Enderby, P.A. (2008) Terminal domains within human MT1 and MT2 melatonin receptors are involved in internalization processes. *J. Pineal Res.* 45, 212–218.
- [17] Manchester, D.X., Terron, L.C. and Petal, M. (2007) One molecule, many derivatives: a never ending interaction of melatonin with reactive oxygen and nitrogen species? *J. Pineal Res.* 42, 28–42.
- [18] Pandi-Perumal, S.R., Trakht, I., Srinivasan, V., Spence, D.W., Maestroni, G.J., Zisapel, N. and Cardinali, D.P. (2008) Physiological effects of melatonin: Role of melatonin receptors and signal transduction pathways. *Prog. Neurobiol.* 85, 335–353.
- [19] Yau, M.Y., Pang, C.S., Kravtsov, G., Pang, S.F. and Shiu, S.Y. (2002) [^{125}I]Iodomelatonin binding sites in guinea pig platelets. *J. Pineal Res.* 32, 97–105.
- [20] Vacas, M.I., Del zar, M.M., Martinuzzo, M. and Cardinali, D.P. (1992) Binding sites for [^3H]-melatonin in human platelets. *J. Pineal Res.* 13, 60–65.
- [21] Regodón, S., Del Prado Míguez, M., Jardín, I., López, J.J., Ramos, A., Paredes, S.D. and Rosado, J.A. (2009) Melatonin, as an adjuvant-like agent, enhances platelet responsiveness. *J. Pineal Res.* 46, 275–285.
- [22] Lopez-Gonzalez, M.A., Calvo, J.R., Osuna, C., Rubio, A. and Guerrero, J.M. (1992) Synergistic action of melatonin and vasoactive intestinal peptide in stimulating cyclic AMP production in human lymphocytes. *J. Pineal Res.* 12, 174–180.
- [23] Marquardt, L., Med, C., Ruf, A., Mansmann, U., Winter, R., Schuler, M., Buggle, F., Mayer, H. and Grau, A.J. (2002) Course of platelet activation markers after ischemic stroke. *Stroke* 33, 2570–2574.
- [24] Bach, A.G., Wolgast, S., Mühlbauer, E. and Peschke, E. (2005) Melatonin stimulates inositol-1,4,5-trisphosphate and calcium release from INS1 insulinoma cells. *J. Pineal Res.* 39, 316–323.
- [25] Castillo-Vaquero, A.D., Salido, G.M. and Gonzalez, A. (2010) Melatonin induces calcium release from CCK-8- and thapsigargin-sensitive cytosolic stores in pancreatic AR42J cells. *J. Pineal Res.* 49, 256–263.
- [26] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.
- [27] Bergmeier, W. and Stefanini, L. (2009) Novel molecules in calcium signaling in platelets. *J. Thromb. Haemost.* 1, 187–190.
- [28] Maruyama, T., Kanaji, T., Nakade, S., Kanno, T. and Mikoshiba, K. (1997) 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins (1,4,5) P_3 -induced calcium release. *J. Biochem.* 122, 498–505.
- [29] Vanacek, J. (1998) Cellular mechanisms of melatonin action. *Physiol. Rev.* 70, 687–721.
- [30] Lerea, K.M., Glomset, J.A. and Krebs, E.G. (1987) Agents that elevate cAMP levels in platelets decrease thrombin binding. *J. Biol. Chem.* 262, 282–288.
- [31] Antigny, F., Jousset, H., König, S. and Frie, M. (2011) Thapsigargin activates Ca^{2+} entry both by store-dependent, STIM1/Orai1-mediated, and store-independent, TRPC3/PLC/PKC-mediated pathways in human endothelial cells. *Cell Calcium.* 49, 115–127.
- [32] Magnier, C., Corvazier, E., Aumont, M.C., Le Jemtel, T.H. and Enouf, J. (1995) Relationship between Rap1 protein phosphorylation and regulation of calcium transport in platelets: a new approach. *Biochem. J.* 310, 469–475.
- [33] Karasek, M. and Winczyk, K. (2006) Melatonin in humans. *J. Physiol. Pharmacol.* 5, 19–39.
- [34] Bourne, R.S., Mills, G.H. and Minelli, C. (2008) Melatonin therapy to improve nocturnal sleep in critically ill patients: encouraging results from a small randomised controlled trial. *Crit. Care* 12.
- [35] Wetterberg, L. (1999) Melatonin and clinical application. *Reprod. Nutr. Dev.* 39, 367–382.
- [36] Citera, G., Arias, M.A., Maldonado-cocco, J.A., Lázaro, M.A., Rosemffet, M.G., Brusco, L.I., Scheines, E.J. and Cardinalli, D.P. (2000) The effect of melatonin in patients with fibromyalgia: a pilot study. *Clin. Rheumatol.* 19, 9–13.
- [37] Jan, J.E. and Freeman, R.D. (2004) Melatonin therapy for circadian rhythm sleep disorders in children with multiple disabilities: what have we learned in the last decade? *Dev. Med. Child Neurol.* 46, 776–782.
- [38] Leger, D., Laudon, M. and Zisapel, N. (2004) Nocturnal 6-sulfatoxymelatonin excretion in insomnia and its relation to the response to melatonin replacement therapy. *Am. J. Med.* 116, 91–95.